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(54) Title: POLYNUCLEOTIDE PROBES ON SOLID SUPPORT THROUGH PHOTO-LABILE LINKAGE

(57) Abstract

Polynucleotide probes bound to solid support through photolabile linkage.

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POLYNUCLEOTIDE PROBES ON SOLID SUPPORT THROUGH PHOTO-LABILE LINKAGE

FIELD OF THE INVENTION

The subject invention relates to polynucleotide hybridization assays and provides polynucleotide probes attached to solid supports through photo-labile linkages.

BACKGROUND OF THE INVENTION

Polynucleotide hybridization assays are used as research and diagnostic tools for the detection and identification of unique or specific polynucleotide sequences in samples of complete, fragmented, or mixed nucleic acid. Various hybridization formats have been developed.

Southern, J. Mol. Biol., 98:503 (1975), discloses a polynucleotide hybridization technique employing radiolabeled nucleic acid probes. This procedure permits autoradiographic detection of probe/analyte hybrids and identification of the polynucleotide sequence of the analyte. However, the Southern procedure, as well as the other diagnostic procedures employing radiolabeled nucleic acid probes, are very complex, time consuming, and have the additional problems and expenses generally associated with radioactive materials such as personnel monitoring and disposal. Thus, such assays have remained a tool of basic research and are not generally employed in applied or commercial areas such as clinical diagnosis.

Ward et al., European Patent Application No. 82301804.9, published June 4, 1982, disclose compositions useful as probes in biomedical research and recombinant DNA technology, wherein said probes comprise purine, 7-deazapurine or pyrimidine covalently coupled to a moiety capable of forming a detectable complex with a polypeptide, said moiety being coupled to purine bases at the 8-position, to deazapurine bases at the 7-position, or to pyrimidine bases at the 5-position to form a modified nucleotide. The resulting modified nucleotides are incorporated into DNA by nick-translation techniques.

Ranki and Soderland, U.S. Patent 4,486,539, disclose a technique for the sandwich hybridization of nucleic acids, said technique

comprising contacting single-stranded nucleic acid from a microorganism with a pair of different nucleic acid reagents, both reagents of the pair being single-stranded and complementary with the microorganism-derived nucleic acid and one of the pair being a nucleic acid fragment attached to a solid carrier, such as a nitrocellulose filter, while the other is a nucleic acid fragment labeled with a radioactive marker, whereby a labeled hybrid is formed attached to the solid carrier, for the identification of a microorganism or group of microorganisms present in a sample. The correctness of the identification is tested by detection of the extent of formation of a labeled hybrid attached to the solid carrier.

Tchen et al., PCT Application No. PCT/FR82/00220, published July 7, 1983, disclose nucleic acid probe compositions which have been chemically modified by the covalent attachment of at least one N-2-acetylaminofluorene group to one of the bases of the nucleic acid. After hybridization with the target homologous nucleic acid sequence, such hybridization may be detected by the use of enzyme-labeled antibodies.

Kourilsky et al., PCT Application No. PCT/FR82/00223, published July 7, 1983, disclose DNA molecules modified by covalent attachment of an oligomer of modified ribonucleotides, or a single modified ribonucleotide, which provides a means for coupling a chemical capable of recognition by another molecule or product.

Co-pending and commonly assigned U.S. Application Serial No. 574,630, discloses polynucleotide probe compositions which contain a polypeptide moiety capable of enzymatically activating a zymogen to initiate a detectable enzymatic reaction cascade.

Falkow et al., U.S. Patent 4,358,535 issued November 9, 1982, disclose a method for detecting the presence of a pathogen in a clinical sample by depositing and fixing said sample on an inert support and hybridizing the genetic material of the target pathogen to a labeled-nucleic acid probe. The label may be a radioisotope, a ligand, a fluorescer, a chemiluminescer, an enzyme, or an antibody.

Kourilsky et al., U.K. Patent Application No. 7913031, published October 31, 1979, disclose a method for detecting the possible

presence of a DNA fragment in a sample comprising the hybridization of the sought fragment with an RNA probe which is coupled to an enzyme either prior to or subsequent to the hybridization reaction. The possible presence of the target nucleic acid sequence is revealed by the action of the enzyme-labeled hybridization product on a chromogen substrate.

Heller, et al., European Patent Application No. 82303701.5, published January 26, 1983, disclose a heterogeneous hybridization diagnostic method which uses luminescer-labeled, single-stranded polynucleotides. After separation of the unhybridized reagent, the sample is exposed to light. Any subsequent light emission is related to the amount of target polynucleotide in the sample. The label may be any of the well known luminescent systems.

Heller et al., European Patent Application No. 82303699.1, published July 14, 1982, disclose a homogeneous light-emitting hybridization assay wherein luminescer-labeled first and second single-stranded reagent segments are hybridized with a complementary target single-stranded polynucleotide from a physiological sample such that nonradioactive energy transfer occurs between the labels of the two reagent segments. At least one of the labels is of the absorber/emitter type such that energy in the form of a photon absorbed from the other light label is re-emitted as a different wavelength. Such secondary emissions can only occur if hybridization has taken place.

Arnold, PCT/US84/01351 published March 14, 1985 discloses a polymeric support system for the synthesis of oligonucleotides featuring universal oxidatively cleaved primers for oligonucleotide synthesis.

Ohtsuka, et al. disclose 2'-O-(o-nitrobenzyl) derivatives of uridine, adenosine and cytidine useful in the synthesis of ribooligonucleotides. The 2'-ether provides 2' protection and such 2'-ether linkage is photo-labile so that the resulting ribooligonucleotide can be collected.

SUMMARY OF THE INVENTION

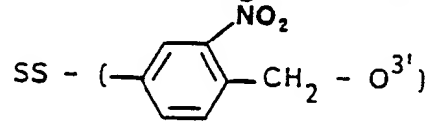
In one aspect, the instant invention provides polynucleotide probes, both RNA and DNA, bound to a solid support through photo-labile linkages. Such support-bound probes may be utilized in conventional sandwich hybridization assays and subsequently cleaved by photolysis for measurement of sandwich pairs. Alternatively, the unhybridized probes may be cleaved by photolysis for subsequent use in any of the well known hybridization assay formats.

In another aspect of this invention, a support-bound photo-labile primer is provided for attachment of polynucleotide probes thereto for subsequent use in sandwich hybridization assays. Alternatively, such solid-support primers can be used in "building" polynucleotide probes either for use in sandwich hybridization assays or for subsequent photo-cleavage thereof for use in any of the well known hybridization assay formats.

The photo-labile linkages of this invention have the advantage of being stable to normal hybridization conditions, as well as to conditions necessary for the synthesis of polynucleotides, e.g., the conventional "blocking" and "de-blocking" steps associated with the protection of exocyclic amino groups and 5'-hydroxyl groups during DNA probe synthesis.

DETAILED DESCRIPTION OF THE INVENTION

The polynucleotide probe of this invention, bound to a solid support through a photo-labile linkage is of the formula

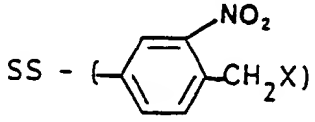


where SS is the solid support, and $O^{3'}$ represents the 3' oxygen of a nucleotide moiety in the polynucleotide probe. Also, the phenyl ring may be naphalene provided that the nitro group is on the ring attached to the 3' oxygen of the nucleotide moiety.

The solid support can be of a number of polymer supports. Examples of such supports are fluoropolymer resins, polystyrene resins, silica, nylon and graft copolymers; and preferred resins

include chloromethylated and bromomethylated resins. These supports are preferably utilized in the form of beads of about 0.1 to 200 microns in diameter.

The support-bound photo-labile primer of this invention is of the formula



where SS is the solid support bound to the phenyl ring as discussed above, and X is a halogen such as Cl or Br. This primer is useful for the synthesis of polynucleotide probes (as exemplified below), or for the attachment of previously synthesized (or natural) polynucleotide probes.

Once the polynucleotide probes are bound to the support through the photo-labile linkage, they can be cleaved by application of UV light having a wavelength of between about 280 nm and 350 nm. As seen in the example below, cleavage takes place between the 3' oxygen and the adjacent benzylic group of the linkage. Of course, when ribonucleotides are utilized, the 2' oxygen is similarly available for such linkage. This cleavage can be carried out simply to collect the polynucleotide probes for later use, or can be carried out following use of the probe as the "capture" probe in a typical sandwich hybridization assay - in which case the "sandwich" is collected for counting. The process for carrying out this sandwich hybridization assay is described in detail in, e.g., U.S. 4,486,539. This invention also allows for a diagnostic kit useful in the detection of a target polynucleotide analyte in a physiological sample wherein the kit comprises a first polynucleotide probe bound to a solid support through a photo-labile linkage, and a second polynucleotide probe, both the first and second probes being substantially complementary to substantially mutually exclusive single-stranded regions of the analyte.

As used herein, the term "target polynucleotide analyte" refers to a segment of single-stranded polynucleotide having a nucleotide base sequence corresponding to a genetic element whose presence in a

physiological sample is to be detected and/or identified; the term "substantially complementary" refers to sufficient nucleotide base sequence homology between a polynucleotide probe and its target analyte to permit formation of stable probe-analtye hybrids; the term "physiological sample" means a sample of blood, urine or other biological tissue, either unprocessed or processed, containing the DNA or RNA of interest; and the term "substantially mutually exclusive" means that upon hybridization by the first and second probes with each target analyte, the two probes should not compete for the same nucleotide base sequence on the analyte to the extent that hybridization is prevented.

Also as used herein, "polynucleotide" refers to a polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), which can be single- or double-stranded, optionally incorporated or comprising synthetic, non-natural, or altered nucleotides capable of incorporation into DNA or RNA polymers. In the case of natural probe polynucleotides, such probes can be conveniently isolated in useful quantities by cloning and amplification of polynucleotide sequences complementary to target polynucleotides in plasmid or phage vectors, using techniques that are now conventional to those skilled in the art. A useful reference covering most aspects of DNA manipulation is Maniatis et al., Molecular Cloning, A Laboratory Manual. (Cold Spring Harbor Laboratory, 1982), the disclosure of which is incorporated herein by reference. An exemplary cloning vehicle for production of useful quantities of probe polynucleotides is plasmid pBR322 (ATCC 37017), which is described in detail by Rodriquez, et al., in Scott, ed., Molecular Cloning of Recombinant DNA, (Academic Press, New York, 1977), p. 72. This plasmid contains single PstI, BamI, EcoRI, HindIII, and SalI restriction endonuclease recognition sites, in addition to genes conferring resistance to the antibiotics tetracycline and ampicillin. Plasmid DNA can be amplified by growth in the presence of chloramphenicol (170 µg/ml) according to the method of Clewell, J. Bacteriol. 110:667 (1972); and purified by the cleared lysate procedure of Guerry et al., J. Bacteriol. 116:1064

(1973), prior to digestion with an appropriate endonuclease. For example, digestion with PstI inactivates the ampicillin resistance marker and generates "sticky ends" suitable for ligation to a probe polynucleotide similarly cleaved with PstI. The resulting recombinant plasmid can then be employed to transform a suitable host bacterium, e.g., E. coli K12 HB101. Upon growth in the presence of chloramphenicol, high plasmid copy numbers can be attained and the recombinant plasmid DNA isolated and purified as previously described. However, a particularly preferred vector for production of probe polynucleotides is a coliphage, M13, (ATCC 15669-BI) which, like pBR322, is now commercially available (New England Nuclear Corporation, Boston, Massachusetts, USA). DNA fragments obtained by digestion of phage DNA and DNA complementary to a target DNA of interest can be joined, amplified, and subsequently purified in single-stranded form prior to conjugation with a reporter molecule, e.g., an enzymatic activator polypeptide such as peroxidase. The use of M13 phage as a cloning vehicle has been described by Messing, Recombinant DNA Tech. Bull. 2:43, (1979), the disclosure of which is hereby incorporated by reference.

In the case of synthetic probe polynucleotides, the synthesis of same is described in the literature. In fact, DNA synthesizers are commercially available. These synthetic probes consist of between about 12 and 500 nucleotide moieties. The base residue can be any purine, modified purine, pyrimidine, or modified pyrimidine base capable of stable incorporation into a single-stranded polynucleotide without significantly affecting the capacity of the polynucleotide to form hybrids with target polynucleotides having substantial complementarity. However, a common feature of all base residue useful in the present invention is a point or points suitable for attachment, preferably covalent, of any of the well known reporting mechanisms. Thus, apart from the "classic" bases adenine, guanine, cytosine, uracil and thymine, other, less common bases, e.g., 5-methylcytosine, 5-hydroxymethylcytosine, orotic acid derivatives, methylated bases, e.g., 1-methylguanine, etc., can optionally be

incorporated into the probes of the present invention. Further, the nucleotides can optionally comprise various substituents, which can be linked to either base or sugar portions, and which do not deleteriously affect the capability of the resulting polynucleotide to form hybrids with complementary target polynucleotides. Polymer "tails" comprising a number of nucleotides appropriate for conjugation to a reporting system can be added to probe polynucleotides by use of calf-thymus terminal deoxynucleotidyl transferase (TdT), which catalyzes the addition of deoxynucleotides to the 3'-hydroxyl ends of single- or double-stranded DNA, as disclosed by Roychoudhury et al., Nucleic Acid Res. 3:101 (1976).

The following Example is an illustration of an application of the subject invention.

EXAMPLE

Nitration of chloromethylated resin:

Synthesis of the o-nitro-chloromethyl polymer proceeds by the procedure of R. B. Merrifield (Journal of the American Chemical Society, 85:2149-2154, 1968). A 5 gm sample of dry chloromethyl polymer (copolystrene-1% divinyl-benzene 5.07m.eq. of Cl/g) is added slowly with stirring to 500 ml. of fuming nitric acid (90% HNO_3 which has been cooled to -10°C). After the addition of all resin, the mixture is stirred at -5°C for 1 hour and then poured into crushed ice. The resin is filtered through sintered glass funnel and washed with water (2 x 500 mls.), dioxane (2 x 300 mls.), and methanol (2 x 200 mls.). The resin is dried under vacuum for 2 hours and stored over Drierite in freezer. Nitrogen analysis of the nitrated resin is done to confirm appropriate nitration of the chloromethylated resin.

Attachment of 5'-O-blocked 2'-deoxynucleoside to o-nitro-chloromethylated resin:

Attachment of 5'-O-blocked nucleoside to o-nitro-chloromethylated resin proceeds by the procedure of E. Ohtsuka, S. Tanaka, M. Ikehara (Synthesis 453-54, July 1977) for the protection of 2'-OH groups of ribonucleosides by o-nitrobenzyl bromide.

Sodium hydride (14.78 mg/440 umoles) is weighed in a serum capped centrifuge tube (15 mls.) and held under Argon atmosphere. Dry dimethylformamide (5 mls.) is added to sodium hydride through the serum cap with a syringe (5 mls.). The suspension of sodium hydride/dimethylformamide is withdrawn with another syringe and transferred to a three-necked flask containing 5'-0-blocked 2'-deoxy nucleoside in dry dimethylformamide (2 mls.) under Argon atmosphere. After hydrogen gas generation has ceased, o-nitrochloromethyl resin (84 mg/440 umoles) is added in small increments. The mixture is allowed to stir at room temperature for 3 hours and then is left standing at room temperature overnight. The resin is filtered through a sintered glass filter funnel and washed successively with dimethylformamide (2 mls. x 2), 90% dimethylformamide in water (2 mls. x 1), and acetonitrile (2 ml. x 2). The resin is then dried in a desiccator under vacuum.

Selective removal of purine/pyrimidine amino-protecting groups, i.e. benzoyl and isobutyryl type:

In order to test whether the ether linkage binding nucleoside to the resin is stable to alkali while the blocking groups are selectively removed from the purine/pyrimidine protecting groups, the dimethoxytrityl nucleoside bound to resin is analyzed in the following manner:

Resin (1-2 mg) is treated with conc. ammonium hydroxide (1 ml.) and allowed to react in a sealed glass ampule in a heating block overnight at 55°C. The resin is centrifuged, and the supernatant (ammonium hydroxide) transferred to another tube. Both the resin and the supernatant are analyzed for the presence of dimethoxytrityl nucleoside based on the liberation of dimethoxytrityl cation when treated without any processing with trichloroacetic in methylene chloride, (supernatant is first evaporated to dryness before treating it with trichloracetic acid in methylene chloride). The presence of most (90-95%) of the dimethoxytrityl cation in resin compared to the supernatant proves that the 5'-dimethoxytrityl blocked nucleoside is

still bound to the nucleoside and thereby confirming that the ether linkage established between the nucleoside and the resin is stable to the alkaline treatment under which the amino blocking groups from purines/pyrimidines are selectively removed.

Synthesis of oligodeoxynucleotide on o-nitrochloromethylated resin:

O-nitrochloromethylated resin is used to synthesize oligodeoxynucleotides either manually or on a DNA synthesizer using phosphoramidite or phosphotriester chemistry. In either case, synthesis begins with the attachment of the first deoxynucleoside (at the 3'-end) to o-nitrochloromethylated resin following the procedure described earlier. The chain is then extended stepwise to the desired length through the 5'-end of the of the oligomer by coupling subsequent nucleoside phosphodiesters or nucleoside phosphoramidites to the 5'-OH end of the preceding nucleoside. Once the desired oligomer is synthesized, it can be subjected to the alkaline treatment (at 55°C) to deblock amino protecting groups of the purine/pyrimidine bases. Under these conditions, the oligomer stays immobilized to support.

Attachment of completely blocked synthetic oligonucleotide to o-nitrochloromethylated resin:

The procedure to attach completely blocked (protected at 5'-OH group and purine/pyrimidine amino groups) oligonucleotides to the o-nitro-chloromethylated resin proceeds by the procedure of attaching a single nucleoside to the resin as described above.

Removal of the synthetic oligonucleotide from photo-labile support:

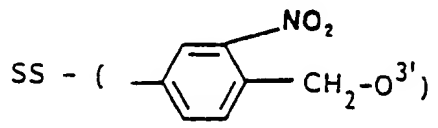
The cleavage of the finished oligonucleotide or the hybridized double stranded oligomer from the photo-labile support can be done by irradiating the sample to 320nm lamp for 4 hours in an RPR-100 apparatus (Ryanote, the Southern Co., Hamden, Connecticut).

CLAIMS

WHAT IS CLAIMED IS:

1. A composition comprising a polynucleotide probe bound to a solid support through a photo-labile linkage.

2. The composition of claim 1 wherein the probe bound to the solid support through a photo-labile linkage is of the formula

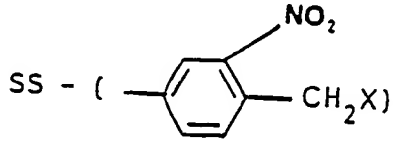


where SS is the solid support and $O^{3'}$ represents the $3'$ oxygen of a nucleotide moiety in the polynucleotide probe.

3. The composition of claim 2 wherein SS is selected from chloromethylated resins, fluoropolymer resins, polystyrene resins, silica, nylon and graft copolymers.

4. The composition of claim 3 wherein SS is a chloromethylated or bromomethylated resin.

5. A composition comprising a support-bound photo-labile primer of the formula



where SS is a solid support and X is a halogen ion.

6. The composition of claim 5 wherein X is Cl or Br.

7. The composition of claim 5 wherein SS is as defined in claim 3.

8. The composition of claim 6 wherein SS is as defined in claim 3.

9. The composition of claim 7 wherein SS is a chloromethylated or bromomethylated resin.

10. The composition of claim 8 wherein SS is a chloromethylated or bromomethylated resin.

11. A kit useful in the detection of a target polynucleotide analyte comprising a first polynucleotide probe bound to a solid support through a photo-labile linkage, and a second polynucleotide probe, both the first and second probes being substantially comple-

mentary to substantially mutually exclusive single-stranded regions of the analyte.

12. The kit of claim 11 wherein the polynucleotide probe bound to a solid support through a photo-labile linkage is of the formula of claim 2.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/00659

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL(4): C12Q 1/68; C07H 21/00
U.S. CL : 435/6; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/6,803,810 935/78; 525/54.11 436/501; 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Computer Search: Lexpat; APS; Chemical Abstracts 1967-1987; Biosis 1977-1987

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
P,X Y	US,A, 4,542,102 (DATTAGUPTA ET AL) 17 September, 1985 see column 3, lines 5-7 and column 6, lines 19-33.	1,11 2-10,12
P,Y	US,A, 4,569,967 (KORNREICH ET AL) 11 February, 1986 see abstract.	3,4,7-10
Y	Journal of the American Chemical Society, Vol. 100, No. 26, issued 20 December 1978 (Washington, D.C. USA) E. Ohtsuka et al, "Studies on Transfer Ribonucleic Acids and Related Compounds. 23. Synthesis of a Heptanucleotide Corresponding to a Eukaryotic Initiator tRNA Loop Sequence" see page 8210.	2-10,12

* Special categories of cited documents: [15]

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IV. CERTIFICATI N

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
7 May 1987	22 MAY 1987
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Jeremy M. Jay

Form PCT/ISA/210 (second sheet) (May 1986)

III. DOCUMENTS CONSIDERED T BE RELEVANT (C NTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
P,Y	Chemical Abstracts, Vol. 103, No. 23, issued 09 December 1985 (Columbus, Ohio, USA) E. Bayer et al "Peptide synthesis on the new polyethene polystyrene graft copolymer synthesis of insulin B_{21-20}" see page 710, column 2, the abstract No. 196383q, Int. J. Pept. Protein Res. 1985 25(2) 178-86 (Eng.)	2-10,12
A	Chemical Abstracts, Vol. 87, No. 19, issued 07 November 1977 (Columbus, Ohio, USA) E.Ohtsuka et al, "Studies on transfer ribonucleic acids and related compounds. XVI. Synthesis of ribooligonucleotides using a photosensitive o-nitrobenzyl protection for the 2'-hydroxyl group." See, page 625, column 2, the abstract No. 152498c, Chem. Pharm. Bull. 1977, 25(5) 949-59 (Eng.)	